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Effects of off-pump versus on-pump coronary artery bypass grafting on function and viability of circulating endothelial progenitor cells

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Objective: Off-pump coronary artery bypass grafting may result in fewer myocardial and vascular complications than on-pump. Although differences in aortic manipulations likely play a role, the systemic responses of endothelial progenitor cells to both types of operations have not been examined. We sought to examine endothelial progenitor cell characteristics after off-pump versus on-pump coronary artery bypass grafting.

Methods: Twenty patients undergoing off-pump or on-pump coronary artery bypass grafting were prospectively enrolled and had endothelial progenitor cells isolated and cultured from their peripheral blood before and 24 hours after surgery. Endothelial progenitor cells were identified by fluorescent dual lectin/low-density lipoprotein binding. Their number, phenotype characteristics, proliferation, migratory function, and viability were determined in a blinded fashion.

Results: Patient characteristics and numbers of grafts were equivalent. Endothelial progenitor cells had similar phenotypes between groups before and after surgery. Off-pump and on-pump coronary artery bypass grafting resulted in similar increases in endothelial progenitor cell numbers and showed equivalent proliferation activity. However, endothelial progenitor cell migratory function was higher in off-pump patients (25.3 $\pm$ 5.0 vs 5.0 $\pm$ 1.0 cells per high-powered field for off-pump vs on-pump coronary artery bypass grafting, respectively; $P = .04$). Postoperative endothelial progenitor cell viability adjusted for preoperative baseline was also higher after off-pump than on-pump coronary artery bypass grafting by 72.4% $\pm$ 14.6% ($P = .01$). Endothelial progenitor cells of on-pump patients were less viable after surgery than before surgery, whereas the reverse was observed in off-pump patients.

Conclusions: Both on-pump and off-pump coronary artery bypass grafting elicit mobilization of endothelial progenitor cells into the peripheral blood. On-pump coronary artery bypass grafting, however, impairs the migratory function and viability of these vascular repair cells, which are conversely preserved after off-pump surgery. Further work is necessary to determine whether the function and viability of endothelial progenitor cells correlate with vascular outcomes and whether their therapeutic modulation may one day benefit coronary artery bypass grafting patients.

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Supported in part by the Canadian Fund for Innovation (Grant 7346, to M. Ruel) and the Heart and Stroke Foundation of Canada (Grant NA5163, to M. Ruel).

Received for publication Oct 29, 2004; revisions received Dec 16, 2004; accepted for publication Jan 10, 2005.

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J Thorac Cardiovasc Surg 2005;130:633-9

0022-5223/$30.00

doi:10.1016/j.jtcvs.2005.01.013

Endothelial progenitor cells (EPCs) are a subtype of circulating bone marrow–derived cells that have the potential to proliferate and differentiate into mature endothelial cells at virtually any site in the body.[1,2] Recent work has indicated that these processes continue to occur in adults and play a role not only in the prevention of endothelial dysfunction and cardiovascular disease progression,[3] but also in the response to inflammation, cardiac

ischemia, myocardial infarction, and other vascular insults.[4-6] EPCs accelerate re-endothelialization and reduce vascular inflammation after vascular injury,[7] and an impaired function of these cells has been correlated with adverse vascular events such as in-stent restenosis and poor collateral development in adults.[8,9]

Vascular trauma, such as trauma that occurs during coronary artery bypass grafting (CABG), leads to a cascade of events that result in vasomotor dysfunction and chemoattraction of leukocytes to cardiac and extracardiac sites. Off-pump coronary artery bypass (OPCAB) has been associated with fewer perioperative vascular complications—including renal dysfunction, stroke, and myocardial infarction—than conventional on-pump CABG,[10-13] and this may relate in part to less aortic manipulation.[14] However, the comparative effects of on-pump CABG versus OPCAB on circulating EPCs remain unknown. Their elucidation may help us to better understand the vascular responses to these 2 types of coronary bypass operations and explain some of the differences observed in myocardial and vascular outcomes. In this study, we examined and compared in a prospective setting the number, phenotype, proliferation activity, migratory function, and viability of EPCs in on-pump CABG and OPCAB patients before and after surgery.

Methods

Patients, Blood Samples, and Surgical Procedures

The study was approved by the Human Research Ethics Board of the University of Ottawa Heart Institute, and informed consent was obtained from all patients. Low-risk patients (n = 20; 10 per group) who had stable, angiographically documented 2- or 3-vessel coronary artery disease and who were scheduled to undergo primary on-pump CABG or OPCAB without another concomitant cardiac surgical procedure were eligible for the study. Exclusion criteria were emergency status, known ascending aortic disease, type 1 diabetes, chronic renal failure, current smoking, cancer, anticoagulation with warfarin, coagulopathy, liver dysfunction, immunomodulating agents such as steroids or other immunosuppressants, administration of granulocyte colony-stimulating factor, recent or active infection, and implantation of an intra-aortic balloon pump or ventricular assist device. All patients received general anesthesia with a routine protocol; on-pump CABG (but not OPCAB) patients also received low-dose intravenous tranexamic acid according to a standardized protocol.

The cell-characterization procedures involved the harvest of 30 mL of fresh blood from a central venous catheter placed in the left jugular vein after anesthetic induction immediately before surgery. Twenty-four hours after surgery, another 30 mL of blood was taken from the same central venous catheter. EPCs were isolated, cultured, positively identified, and counted; their phenotype was examined; and their function was evaluated with proliferation, migration, and viability assays as described below.

The surgical procedures within each study group were performed by the same 2 surgeons, each with a CABG caseload that involved the use of beating-heart revascularization for approximately 50% of cases. The surgeon's preference of on-pump CABG versus OPCAB in each patient was not dictated by protocol and had been determined for all patients before entry into the study. On-pump operations were performed with standard heparin management and by using cold crystalloid cardioplegia. OPCAB operations used a heparin dose of 150 U/kg intravenously, adjusted to reach a target activated clotting time of 300 seconds during the grafting period, and were performed by using an apical suction device and epicardial coronary stabilizer (Medtronic, Toronto, Canada). After surgery, on-pump and OPCAB patients received 650 mg of acetylsalicylic acid within 6 hours of operation.

Cell Isolation and Culture

Total peripheral blood mononuclear cells (MNCs) were isolated from fresh blood samples by Histopaque 1077 (Sigma-Aldrich, Oakville, Canada) density-gradient centrifugation of buffy coats. One million MNCs per square centimeter were plated on fibronectin-coated tissue culture flasks and cultured in endothelial basal medium (EBM-2; Clonetics, Guelph, Canada) supplemented with EGM-2-MV-SingleQuots (Clonetics) containing 5% fetal bovine serum, 50 ng/mL human vascular endothelial growth factor (VEGF), 50 ng/mL human insulin-like growth factor 1, and 50 ng/mL human epidermal growth factor. After 4 days in culture, nonadherent cells were removed by washing, new medium was applied, and the culture was maintained through day 4. To confirm the EPC phenotype, direct fluorescent staining was used to detect dual binding of fluorescein isothiocyanate (FITC)-labeled *Ulex europaeus* agglutinin 1 (ulex-lectin; Sigma, Oakville, Canada) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)-labeled acetylated low-density lipoprotein (acLDL; Molecular Probes, Burlington, Canada) on attached adherent cells in day 4 culture. Cells were visualized with a fluorescent microscope, and adherent cells that stained positive for both FITC–ulex-lectin and DiI-acLDL were considered to be EPCs.[15] Ten randomly selected high-powered fields (HPFs) from 2 individual wells were examined in each sample, and the number of dual-staining cells was counted by an observer blinded to the patient's study group.

Flow Cytometry

To examine the EPC phenotype, detached cells were examined by flow cytometry. Cells were labeled for 20 minutes at manufacturer-recommended concentrations with the following fluorescent antibodies: anti-CD34 (a stem/progenitor cell marker detected on cultured EPCs), anti–vascular endothelial (VE) cadherin (an endothelial lineage marker), and anti–VEGF receptor 2 (VEGF-R2; also an endothelial lineage marker). Samples were analyzed in a blinded fashion by using a Beckman-Coulter flow cytometer (Beckman, Mississauga, Canada).

Functional Assays

To examine the proliferation potential and function of cultured EPCs, proliferation, migration, and viability assays were performed on cells after 7 days (proliferation and migration) and 4 days (viability) of culture by using the conditions described previously. Previous reports have demonstrated maintenance after in vitro incubation of the phenotypic differences of EPCs at the time of harvest.[16-20]

Proliferation. For the proliferation assay, 7-day-cultured EPCs were lifted by pipetting vigorously, replated onto 96-well plates precoated with fibronectin, and serum-depleted for 24 hours. After 24 hours, EPCs were supplemented with 10 μL methyl-thiazol-diphenyl-tetrazolium (5 g/L; Sigma) and incubated for another 4 hours. The supernatant was discarded by aspiration, the EPC preparation was shaken with 200 μL of dimethyl sulfoxide for 10 minutes, and the solution's optical density was measured at 490 nm.

Migration. For determination of cell migration, isolated cells were detached by vigorous pipetting, harvested by centrifugation, resuspended in 500 μL of EBM-2 with supplements, and counted. Then, 2×10^4 EPCs were placed in the upper chamber of a modified Boyden chamber (Hemogenix, Colorado Springs, Colo) while VEGF (10 ng) in serum-free media was placed in the lower chamber. After 24 hours of incubation at 37°C, cells were stained for quantification with the fluorescent dye carboxifluorescein diacetate. Cells that migrated into the lower chamber were counted manually in 3 random HPFs by a blinded observer. As a negative control, EPC migration was also compared with that of healthy volunteers (n = 3).

Viability. Cell viability was determined with the trypan blue exclusion method. When cells die, their membrane becomes permeable and allows uptake of the trypan blue dye, and nonviable/dead cells become darker than the viable cells. To determine nonviability, fresh blood EPCs and EPCs cultured for 4 days in a 35-mm fibronectin-coated dish were detached with phosphate-buffered saline with 1 mmol/L ethylenediaminetetraacetic acid, followed by gentle shifting, and were resuspended after centrifugation in a total volume of 2.0 mL per dish. Nonviable cells were counted from starting random samples of 3×10^6 cells in triplicate in an automated fashion by using a Vi-Cell Beckman-Coulter counter (Beckman).

Statistical Analysis

Values are expressed as mean ± standard error of the mean. Statistical analyses were performed in Intercooled Stata 8 (Stata, College Station, Tex). Mean postoperative-preoperative EPC nonviability ratios were calculated for each patient as follows: postoperative-preoperative EPC nonviability ratio = (postoperative percentage of nonviable cells − preoperative percentage of nonviable cells)/preoperative percentage of nonviable cells × 100%. Comparisons of continuous data between groups were performed with analysis of variance. Dichotomous variables were compared within groups by using the Fisher exact test.

Results

Patient Characteristics and Outcomes

Table 1 displays the characteristics of the patients enrolled in the study. There were no crossovers or intraoperative conversions between the on-pump CABG and OPCAB groups, and there were no significant differences in preoperative characteristics, the number of grafts, or the incidence of postoperative complications. Mean cardiopulmonary bypass and aortic crossclamp times in on-pump patients were 69.7 ± 5.5 minutes and 45.5 ± 4.8 minutes, respectively. All patients were free from readmission or major adverse cardiac events at the 1-month follow-up.

TABLE 1. Patient characteristics and operative variables

Variable	On-pump CABG (n = 10)	OPCAB (n = 10)
Female sex	1	1
Age, y (mean ± SEM)	58.3 ± 2.7	63.3 ± 4.3
Body surface area (m^2)	2.0 ± 0.1	1.8 ± 0.1
Left ventricular ejection fraction <40%	2	2
Hypertension	5	4
Type 2 diabetes	0	2
Preoperative serum creatinine, μmol/L (mean ± SEM)	101.7 ± 15.8	107.5 ± 15.9
No. grafts (mean ± SEM)	2.5 ± 0.2	2.5 ± 0.2
Blood transfusion	3	3
Reopening for bleeding	0	0
Myocardial infarction, stroke, or death	0	0
Hospital length of stay, d (mean ± SEM)	6.2 ± 1.7	4.9 ± 0.6

CABG, Coronary artery bypass grafting; *OPCAB,* off-pump CABG; *SEM,* standard error of the mean.

Cell Numbers and Phenotype

After 4 days in culture, MNCs isolated from the patients' blood that exhibited a spindle-shaped endothelial cell–like morphology, adherence, and positive staining for both DiI-acLDL uptake and lectin binding were characterized as EPCs (Figure 1). At 24 hours after surgery, on-pump CABG and OPCAB resulted in an increase in the number of circulating EPCs of 54.3% ± 32.2% and 44.1% ± 17.2%, respectively, compared with the preoperative baseline (P = .88 between the on-pump and OPCAB groups). Flow cytometry analysis revealed that EPCs of on-pump CABG versus OPCAB patients had similar phenotypes before and after surgery, with no significant difference in the expression of CD34, VE cadherin, or VEGF-R2 at any study time point between the on-pump and OPCAB patients. Overall, CD34, VE cadherin, and VEGF-R2 markers were expressed in 45.4% ± 3.8%, 95.0% ± 1.2%, and 90.2% ± 3.0% of cells, respectively.

Proliferation

There was no difference in postoperative proliferation activity between EPCs harvested from on-pump CABG versus OPCAB patients. Proliferation activity measured by light absorbance at 490 nm was 0.154 ± .015 versus 0.157 ± .008 in the on-pump versus the OPCAB group, respectively (P = .9).

Migration

Boyden chamber assays revealed increased postoperative EPC migration in OPCAB patients versus on-pump CABG

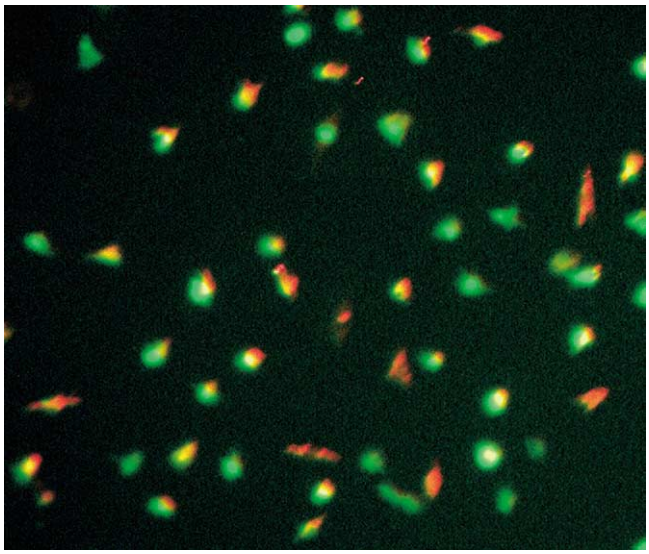


Figure 1. Endothelial progenitor cells 24 hours after surgery (original magnification, 400×). Mononuclear cells isolated from the peripheral blood (in this example, from an OPCAB patient) were characterized as endothelial progenitor cells if they exhibited spindle-shape endothelial cell–like morphology and stained double-positive for lectin binding *(green)* and labeled acetylated low-density lipoprotein *(red)*.

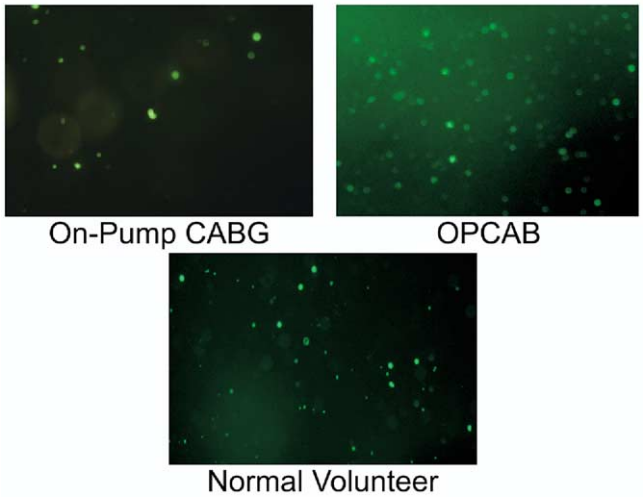


Figure 2. Migration assays of cultured human EPCs (original magnification, 100×). Photomicrographs of day 7 cultured EPCs harvested 24 hours after surgery from on-pump CABG and OPCAB patients. There was improved EPC migration in samples from the OPCAB compared with the on-pump patients ($P = .04$; see text). Also shown are day 7 cultured EPCs harvested from a healthy volunteer who did not undergo operation. The postoperative EPC migratory activity of OPCAB patients was also higher than that of healthy volunteers, but this difference was not statistically significant ($P = .05$; see text).

patients (Figure 2); migration was 25.3 ± 5.0 cells per HPF in OPCAB patients, versus 5.0 ± 1.0 cells per HPF in on-pump CABG patients ($P = .04$). the EPC migratory activity of OPCAB patients was also higher than that of healthy volunteers, who exhibited a cell migration capacity of 16.0 ± 6.1 cells per HPF, but the difference was not statistically significant ($P = .05$).

Viability

Details of viability analyses are presented in Figure 3. Before surgery, there was no significant difference in EPC nonviability between the on-pump CABG and OPCAB groups (9.7% ± 1.0% vs 12.3% ± 2.0% of nonviable cells before surgery for the on-pump and OPCAB groups, respectively; $P = .2$). However, 24 hours after surgery, the number of dead/nonviable EPCs had increased to 13.4% ± 1.0% in the on-pump CABG group, whereas the number of nonviable cells had decreased to 8.4% ± 0.4% in the OPCAB group ($P < .001$ vs the on-pump CABG group). Furthermore, the mean of individual postoperative-preoperative EPC nonviability ratios was +58.7% ± 22.3% for on-pump CABG patients; in contrast, OPCAB patients demonstrated a negative (−13.8% ± 14.6%) mean postoperative-preoperative EPC nonviability ratio ($P = .02$ vs the on-pump group). Overall, the mean cumulative difference in postoperative-preoperative EPC nonviability ratios between the on-pump and OPCAB groups was 72.4% ± 17.7%, in favor of the OPCAB group.

Discussion

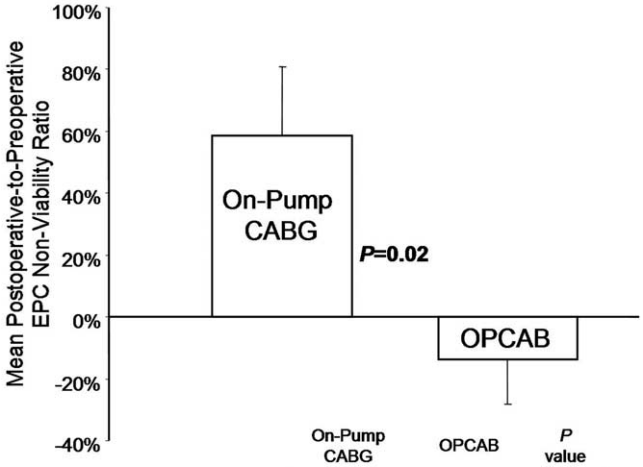
Main Findings

The main findings of this study were that (1) CABG performed on cardiopulmonary bypass, although it results in an increased number of postoperative EPCs, in accordance with previous work,[21,22] significantly impairs the migratory capacity and viability of EPCs after surgery, as evidenced by significant differences found in EPCs harvested from on-pump CABG patients 24 hours after surgery. These changes were significant not only compared with a matched control OPCAB group population, but also, in the case of viability assays, compared with preoperative baseline, ie, using each on-pump CABG patient as his or her own control. In addition, the data from this study indicate that (2) OPCAB, like on-pump CABG, increases circulating EPC numbers after surgery and that (3) OPCAB, in contrast to on-pump CABG, preserves the viability and migratory function of EPCs after surgery.

Clinical Implications

The implications of these findings are potentially multiple yet speculative at present. In a growing body of evidence

suggesting that EPCs play a role in preventing vascular events and abnormal vascular healing, this study provides the first available data on the functional response of EPCs to on-pump CABG, as well as the first comparison of these responses to those encountered after OPCAB. These findings suggest that the different functional responses of EPCs may constitute an additional biological basis, in addition to known mechanical and surface-related factors, such as aortic cannulation, clamping, and platelet/leukocyte activation, in explaining the higher rate of multisystemic vascular complications with on-pump CABG versus OPCAB reported in the literature.[10-13] In this regard, a notable discrepancy exists between reports of a trend or a significantly lower incidence of perioperative myocardial infarction with OPCAB versus on-pump CABG in systematic reviews to date,[10,12,13] despite findings of lower angiographic graft patency with OPCAB in 2 of 3 large randomized controlled trials.[23-25] This study was not, however, powered to verify whether EPC functional responses account for this discrepancy nor to verify whether a correlation exists between clinical vascular events and postoperative EPC function between and within on-pump CABG and OPCAB patient groups. It was also not designed to determine for how long the differences persist in EPC viability and function between on-pump CABG and CABG herein identified at 24 hours after surgery. The present findings nevertheless provide a rationale for future work to elucidate these questions; this could, in turn, subsequently justify investigating the role of EPC-modulating agents such as granulocyte macrophage-colony growth factor[18] in decreasing myocardial and extracardiac vascular complications after on-pump CABG and possibly other cardiac operations that involve cardiopulmonary bypass.



	On-Pump CABG	OPCAB	*P* value
Preoperatively	9.7±1.0%	12.3±2.0%	0.2
24h Postoperatively	13.4±1.0%	8.4±0.4%	<0.001
Post-to-Pre Increase in Non-Viability	+3.7±1.6%	-3.9±2.1%	0.02
Post-to-Pre EPC Non-Viability Ratio	+58.7±22.3%	-13.8±14.6%	0.02

Figure 3. Endothelial progenitor cell (EPC) nonviability after on-pump CABG versus OPCAB. On-pump CABG and OPCAB had no significant difference in EPC viability before surgery. However, postoperative viability, the absolute increase in nonviability, and the mean postoperative-preoperative EPC nonviability ratio were significantly higher in patients who underwent on-pump CABG than in those who underwent OPCAB.

Possible Mechanistic Correlates

Migration. The EPCs cultured from OPCAB patients demonstrated an improved ability to migrate compared with those obtained from on-pump CABG patients. No differences in EPC expression of VE cadherin (an adhesion protein) or VEGF-R2 were, however, observed between OPCAB and CABG patients, and it therefore seems unlikely that these would have contributed to the observed difference in migratory potential. Among other candidates are integrins (including β_1 and β_2), which are involved in EPC adhesion and migration[26] and whose integrin-linked kinase and subsequent survival kinase Akt activity promotes endothelial cell migration, survival, and cardiac repair.[27] Another family of proteins involved in the regulation of migration through the extracellular environment are the matrix metalloproteinases (MMPs), which are proteases secreted by migrating cells. Upregulation of MMP-9 expression is required for the recruitment of EPCs from the bone marrow from their quiescent to their activated state.[28] However, the effects of MMPs on the characteristics of EPCs and on determining their migratory potential are currently unknown and represent, like that of integrin expression, a new focus for investigation.

Viability. There was a significant difference in the viability of EPCs from CABG and OPCAB patients. Although equipped with antioxidative stress-associated genes, EPCs can be affected by oxidative stress, with an associated increase in apoptosis.[29] Because OPCAB is associated with less oxidative stress than on-pump CABG,[30] it is possible that the enhanced viability of EPCs in OPCAB patients is related to this mechanism.

Limitations

Controversy exists as to what truly constitutes a circulating EPC, because cell types reported as EPCs in the literature vary greatly. Although most investigators use dual binding of FITC–ulex-lectin and DiI-acLDL, as well as phenotype characteristics of endothelial cells, to define EPCs,[15] pluripotent blood monocytes may actually constitute 1 of 2 types of so-called EPCs described in the human peripheral blood.[2] The functional difference between the 2 EPC populations, which may be distinguished by their proliferating potential

and viability after 4 to 6 weeks in in vitro culture, is unclear because both cell types seem to play a role in neovascularization and re-endothelialization and have the ability to differentiate into endothelial cells.

The study was not randomized, and although preoperative patient characteristics and intraoperative data were balanced between the 2 groups, it remains possible that unknown confounders affected the results. The number of patients did not allow for a multivariate determination of other potential risk factors for EPC nonviability/dysfunction, such as age or diabetes mellitus, which were respectively lower and less prevalent, albeit not significantly, in the on-pump CABG versus the OPCAB group. These small differences, equally unlikely to be have been avoided in a randomized study of similar size, may nevertheless have resulted in the apparent yet nonsignificant difference in preoperative EPC nonviability between the on-pump CABG and OPCAB groups. To minimize the effect of these potential biologic imbalances, all analyses of EPC number and viability in this study were adjusted with respect to preoperative baseline, and assessments of EPC migratory function were compared with those of healthy volunteers.

Conclusions

Despite these limitations, this study provides new insight into the biological responses to on-pump CABG, which results in decreased EPC migratory function and EPC viability, as well as to OPCAB, which does not lead to either of these effects. Although the implications of these findings are still incompletely understood, previous work that has linked EPCs with the prevention of vascular events and the promotion of normal vascular healing suggests that these responses could play a role in the pathogenesis of a higher rate of vascular complications after on-pump CABG compared with OPCAB. These data also provide a rationale for future work examining whether EPC responses after cardiac operations correlate with vascular events and healing and the subsequent investigation of the possible role of therapeutic interventions that could modify the response of EPCs after cardiac surgery.

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